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Short communication

Capillary electrophoretic assay for nitrate levels in the vitreous of proliferative diabetic retinopathy

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Abstract

The determination of nitric oxide (NO) in human vitreous samples is complicated by the relatively short half-life of the analyte and the viscous, high salt and protein biological matrix. In this work, we developed a fast (<5 min) and useful CE method to determine the stable metabolite, nitrate, from vitreous samples. This proposed method has been successfully applied to determine the nitrate levels from the vitreous humor of patients undergoing vitrectomy for a variety of conditions. A statistically significant increase ($P = 0.000001$) of the mean level of nitrate was observed in vitreous humor of patients with proliferative diabetic retinopathy (41.17 ± 4.09 μM, $n = 27$) versus controls (15.22 ± 0.86 μM, $n = 35$). The elevated levels of nitrate in the vitreous of patients known to have diabetic retinopathy suggests that NO is involved with the pathology of this disease.

Keywords: Nitric oxide; PDR; Capillary electrophoresis; Clinical samples

1. Introduction

Nitric oxide (NO) is recognized as an important mediator in the eye [1,2] and it is required for the proper functioning of the retina [3,4]. NO plays significant roles in signal transduction among horizontal [5] and amacrine cells [6] as well as in causing retinal vessel dilation [7], it is also implicated in ganglion cell death [8,9] at high levels. There is evidence shown in recent studies [10–13] that NO contributes to the pathogenesis of proliferative diabetic retinopathy (PDR), which is a leading cause of blindness. Therefore, it is of great importance to develop reliable methods that can be used to monitor vitreous NO levels. The vitreous NO levels of PDR patients can then be compared to those of non-diabetic patients.

A stable metabolite of NO is nitrate and it is generally accepted as an indicator of NO [14,15]. This indirect measurement is generally used because NO has a very short half-life in biological systems [16,17]. Most studies of NO concentrations from vitreous samples are conducted by detection of nitrate using the Griess reaction [12,13,18,19]. Briefly, nitrate levels from vitreous samples are quantified by the chemical reduction of nitrate to nitrite, and then the reaction of nitrite with the Griess reagents produces a purple azo dye which can be measured at 540 nm. However, this method is time consuming, a series of complicated chemical reactions are needed, and the yield of the chromophore is dependent on the order of reagents added. Another available analytical method is the direct measurement of NO using a chemiluminescence NO analyzer [11]. While there are advantages to a direct measurement, the instrumentation is relatively expensive and, due to the short half-life of NO, this instrument must be placed near to the source of the vitreous samples.

CE is recognized as an important analytical technique for its speed, efficiency, reproducibility, small sample volume compatibility, low cost and low consumption of solvent. The use of CE has been described for the determination of NO metabolites by several authors [20,21,22,23]. Cruz et al. [20] have performed the CE-UV analyses of NO metabolites from individual molluscan neurons. Leone et al. [22] reported the measurement

Abbreviations: PDR, proliferative diabetic retinopathy; MH, macular hole; ERM, epiretinal membrane; KRB, Krebb's–Ringer Buffer

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of nitrate and nitrite in plasma by CE-UV detection. Fukushi et al. [21] reported the determination of nitrate and nitrite in environment waters by using CE with on-column transient isotachophoresis. The same group [23] later developed a run buffer which was suitable for the determination of NO metabolites in human serum by CE. We developed a fast and simple method for the direct detection of NO metabolites in rat striatum using CE-UV detection [24]. CE has also been used for the analyses of potassium [25] and amino acids [26] from vitreous samples. In this paper, we demonstrate a fast CE analysis of nitrate from vitreous fluid. This method provides the possibility of using CE for clinically relevant vitreous samples, and we demonstrate its use by comparing vitreous nitrate levels in PDR patients with non-diabetic patients.

2. Experimental

2.1. Chemicals

Citric acid anhydrous, sodium citrate (enzyme grade), anhydrous calcium chloride, magnesium chloride, sodium phosphate monobasic, sodium phosphate and sodium hydroxide were purchased from Fisher Scientific (Itasca, IL); they were of Certified ACS grade, unless specified otherwise. Sodium nitrite (99.999%), sodium nitrate (99.995%), potassium chloride, sodium chloride, cetyltrimethylammonium chloride (CTAC, ≥98%) were from Sigma–Aldrich (St. Louis, MO), they were of SigmaUltra grade, unless specified otherwise. All solutions were prepared with ultrafiltered, deionized water from a US Filter Purelab Plus purification system (Lowell, MA). The composition of the Kreb's–Ringer Buffer (KRB) was 3 mM KCl, 145 mM NaCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.61 mM $NaHPO_4$, and 0.4 mM NaH_2PO_4 [27]. Nitrite and nitrate stock solutions were prepared in the deionized water, and appropriate dilutions were made with either KRB solution or run buffer to the appropriate concentrations.

2.2. Vitreous sample preparation

Vitreous samples were collected under approved Institutional Review Board protocols from patients already undergoing vitrectomy. Volumes of 200 μL undiluted vitreous were pipetted into centrifuge tubes that contained 100 μL 1 M acetic acid (pH 2–3) in KRB to deactivate proteases for concurrent analyses of amino acids in the lab. Vitreous samples of patients were delivered to the University of Illinois at Chicago by overnight courier. Upon arrival samples were refrigerated at 4 °C and all samples were examined within 2 weeks unless specified otherwise.

2.3. Instrumentation

NO was measured by detection of a stable metabolite, nitrate, using a laboratory-built CE system. The CE capillary, buffers, and electrode assembly were placed in a plexiglass box to isolate these components and protect the operator from the high voltage. A VUV-20 variable wavelength ultraviolet/visible absorbance detector (Hyperquan, CO) was used at 214 nm. Control of the high voltage power supply (Spellman, NY) and data acquisition were achieved using a multifunction interface board and a routine written in Labview (National Instruments, TX). Briefly, separations were performed in a 360 μm O.D. × 75 μm I.D. fused silica capillary (BioTAQ, MD) at an applied voltage of −5 kV unless otherwise noted. The total capillary length was 40 cm and the effective length was 22 cm. The optimized separation buffer consisted of 150 mM sodium chloride with 2 mM CTAC adjusted to pH of 3.5 with 5 mM sodium citrate and citric acid. Unless specified otherwise, the samples were injected hydrodynamically at 10 cm for 10 s. The absorbance was read at a frequency of 10 Hz and stored in text format.

2.4. Data analysis

Raw data were exported by Labview (National Instruments, TX) into Excel (Microsoft Corporation, CA) for plotting electropherograms. The baseline was considered to be the average detector signal for 2 s before the nitrate peak, and 2 s after the peak and the noise was taken as the standard deviation of the same section of the electropherogram. The S/N was calculated from the peak height minus the baseline divided by standard deviation of the baseline.

The method of standard additions was used to minimize any vitreous matrix effect that would complicate quantitation. A 5 μL of the vitreous sample was added to each of five microcentrifuge tubes. Then 0–4 μL aliquots from a 40 μM standard nitrite and nitrate stock solution were added. Finally, each tube was made up to a total volume of 10 μL with run buffer and mixed well.

The statistical calculations were performed in Excel. An *F*-test was first performed to determine whether two standard deviations from PDR group and control group were significantly different. Student's *t*-test was used to compare nitrate concentrations between PDR patients and patients from control groups; two-sample unequal variance was selected for the *t*-test according to the previous *F*-test; and the results were expressed as mean ± standard error in the mean. Levels of statistical significance were set at $P < 0.05$.

3. Results and discussion

3.1. Nitrate measurement

In our previous published work [24], optimal CE separations were achieved with 20 mM phosphate, 2 mM CTAC buffer at pH 3.5. In our later work in the development of an automated injection device for the determination of nitrite and nitrate [28], citrate buffer was found out to have better buffer capacity, but no biological samples were tested. The vitreous samples like most of the biological samples contain a high salt content; therefore, the running buffer is modified to have an electrolyte composition similar to the sample. A total of 150 mM NaCl is added to the buffer solution and citrate/citric is used to adjust the pH. The run buffer also contained the cationic surfactant CTAC to reverse

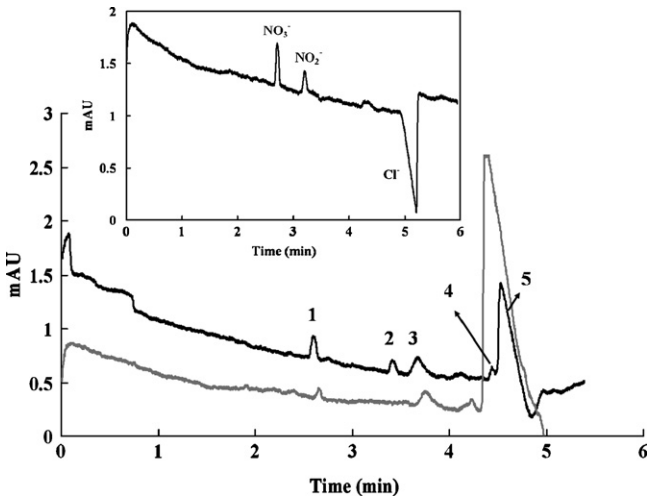

Fig. 1. Representative electropherograms of vitreous samples (diluted 1:1 with the run buffer) from both PDR (upper trace) and ERM (lower trace) patients. Peak 1 was identified as nitrate, peak 5 is identified as acetic acid, and peaks 2, 3, and 4 are not identified. Experimental conditions: 40 cm × 75 μm I.D. fused silica capillary, UV detector at 214 nm, −125 V/cm separation field strength, 10 cm/10 s gravity injection, run buffer: 150 mM NaCl with 2 mM CTAC, pH adjusted to 3.5 by 5 mM citric acid/citrate. Inset is the electropherogram of 32 μM nitrate and 32 μM nitrite standard solution.

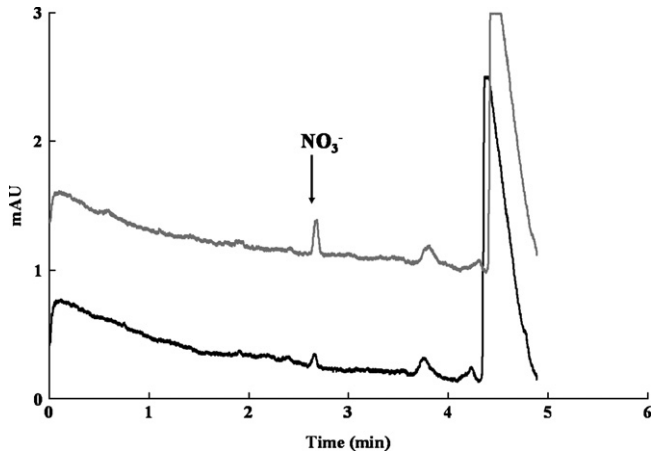

Fig. 2. Representative electropherograms of a vitreous sample (lower trace) and the sample that spiked 16 μM standard nitrate (upper trace) of one patient. CE conditions same as Fig. 1.

the direction of electroosmotic flow to match the direction of the anion migration in the capillary. A representative electropherogram of standard nitrite and nitrate separation is shown as the inset of Fig. 1. There is a negative chloride peak separated from nitrite and nitrate; and this negative peak is due to the less chloride in standard solution compared with total chloride combined from both 150 mM NaCl and 2 mM CTAC in the run buffer.

A total of 100 μL 1 M acetic acid in KRB solution is added to the 200 μL vitreous sample to deactivate proteases for concurrent analyses of amino acids in the lab. The effect of 1 M acetic acid was tested. There is no interference with the determination of nitrate, as shown in Fig. 1, electropherograms of vitreous samples. The peak due to acetic acid migrates past the detection window well after the nitrate peak. Shown in Fig. 2 are the representative electropherograms of a vitreous sample and that sample spiked with 16 μM nitrate standard. This confirms that the measured signal is due to the nitrate.

For the preliminary tests, a calibration curve of nitrate standard solutions is set up and used for the quantification. However, the chemical composition of the vitreous is somewhat complicated. It contains high protein content; it is viscous due to hyaluronic acid; and like other biological samples it also contains about 0.15 M salt content. In order to more accurately quantify the nitrate level in such a complex matrix, the standard addition method is used instead to measure the nitrate levels from vitreous samples. A series of standard additions are performed. The standard addition curve is plotted, and a linear regression is fitted to the points. Shown here is the example of the calculation of the nitrate level from one vitreous sample. The representative equation of one vitreous sample is expressed as $y = 3.444x + 21.15$ ($r^2 = 0.9977$). The absolute value of the x-intercept, which is 6.141, is multiplied by 2 for standard addition dilution correction and by 1.5 considering the vitreous was diluted by acetic acid. The measured nitrate concentration is then 18.42 ± 1.80 μM for this example. A comparison of the two quantification methods shows that the calibration curve generates a value 50% larger than those obtained using the standard addition method. The results from the standard additions match both values obtained by the Griess reaction [12] and direct NO chemiluminescence [11] reasonably well. The determination appears adversely affected by the vitreous matrix so the reported values are calculated from standard additions.

3.2. Stability of the samples

One of the potential advantages with the method here is the measurement of the more stable nitrate anion. The veracity of this claim is tested. Vitreous samples were collected in Minnesota and West Virginia and shipped to University of Illinois at Chicago by overnight courier. A number of vitreous samples were tested by comparing the CE results from samples run shortly after receipt versus samples reanalyzed at later time points. We retested samples 1 week, 2 weeks, 1 month and 2 months later. We found out that the vitreous samples were stable and not degraded within a 1-month period. Shown in Fig. 3(A) is the comparison of the electropherograms at initial and 1 month time points. Both the peak height and the peak shape of nitrate peak remained the same. For a sample analyzed after 2 months, all the peaks include the nitrate were lower, as shown in Fig. 3(B). These results clearly demonstrate that the analysis of nitrate in vitreous samples is stable within 1 month, and the shipping and storage is not deleterious to our analysis. This would be one of the advantages of using a CE assay for the analysis of stable NO metabolites. The intra-day peak height reproducibility (relative standard deviation) of a same vitreous sample was found to range from 1 to 4% ($n = 3$). Also the stability tests showed that within 1 month the peak height was within the intra-day reproducibility.

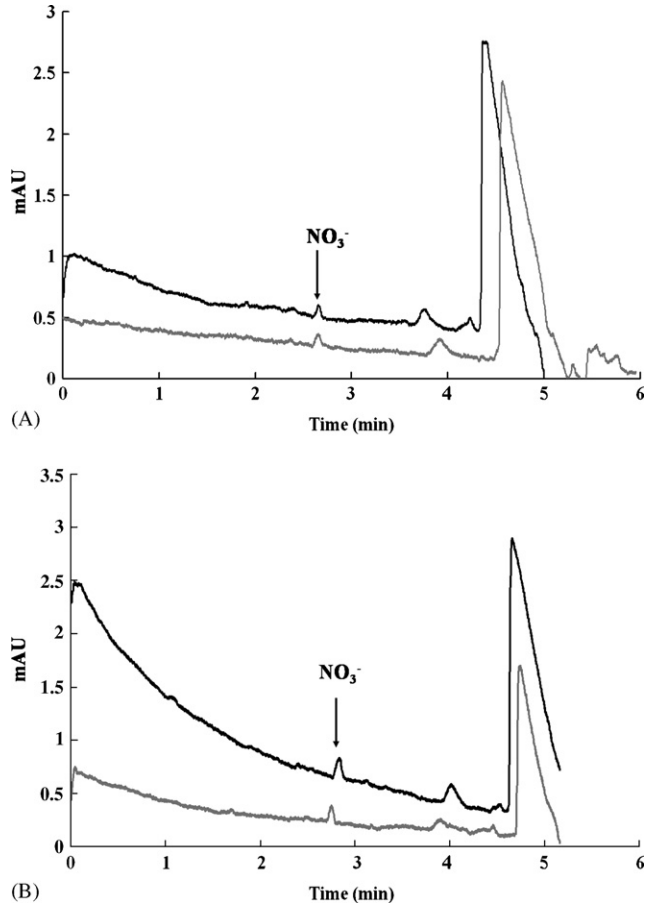

Fig. 3. (A) Comparison of electropherograms of a vitreous sample injected upon receipt (upper trace) and the same sample reassessed 1 month later (lower trace); the nitrate peak varied by 1.4%. (B) Comparison of electropherograms of a vitreous sample injected upon receipt (upper trace) and the same sample reassessed 2 month later (lower trace); the nitrate peak height decreased 18%. Experimental conditions are same as Fig. 1.

3.3. Vitreous samples analyses

With the characterization of the method we turned to the measurement of nitrate in the vitreous samples. This study included 27 patients with PDR, and 35 non-diabetic patients who with other conditions requiring vitrectomy served as a control group. PDR is characterized by abnormal vessel growth into the retina or optic nerve induced by ocular ischemia and fibrosis, which ultimately leads to vitreous hemorrhage and tractional retinal detachment [29]. In the control group the diagnosis included macular hole (MH) which is a small break in the macula located in the retina and epiretinal membrane (ERM) which is a condition characterized by growth of a membrane across the central retina of the eye. Shown in Fig. 1 are representative electropherograms of one control patient and one PDR patient. Comparing the electropherograms from the two different types of diseases, we found out that the pattern of PDR electropherograms is somewhat more complicated than the control one. There is one more peak (peak 2) in the electropherogram from the PDR patient that is not present with non-diabetic patients. Peak 1 in both of the electropherograms was identified as nitrate, and peak 5 was the acetic acid peak, which does not interfere with nitrate analysis. Several peaks (peaks 2, 3 and 4) were not identified; these are interesting targets for further study. One interesting possibility is that one peak may correspond to nitrotyrosine [30,31].

Importantly, the nitrate peak is observed to be significantly higher in PDR patients than in the non-diabetic patients. The average nitrate concentration in PDR group ($n = 27$) is statistically higher than in control ($n = 35$) group (41.17 ± 4.09 μM versus 15.22 ± 0.86 μM, $P = 0.000001$). Our findings of elevated nitrate level in PDR patients are very similar compared with other literature reports [12,19,13]. The previously reported levels in PDR patients ranged from 31.6 ± 2.96 to 49.8 ± 5.0 μM, and ranged from 15.9 ± 1.4 to 24.2 ± 2.8 μM in controls. The source of this NO is not clear. NO is synthesized from L-arginine by various nitric oxide synthase (NOS) enzymes. There are three different isoforms of NOS which may be involved with the results here. The neuronal isoform is activated by calcium influx, which is activated by glutamate stimulation, and glutamate has been shown to be elevated in PDR [32]. In PDR patients, there are abnormal new blood vessels grow on the surface of the retina; there is evidence that these vessels can leak [33]. There is a report that increased NO levels in vitreous may relate to plasma levels [19]. In addition, it has been recently reported that inducible NOS, rather than endothelial NOS, may play a role in the pathogenesis of neovascularization [34]. The inducible isoform has also been implicated in degeneration of retinal ganglion cells seen with diabetic retinopathy [35,36]. While the exact source of our observed nitrate level still needs to be determined, the significantly elevated nitrate level in the vitreous of patients with PDR suggests NO may play a role in the pathology of PDR.

The developed CE method has a detection limit of nitrite of about 4 μM. The nitrite level in vitreous samples is reportedly very low [13,18]. It is not detected by Oku et al. [13] and they estimated it to be less than 1 μM. On the other hand, Diederen et al. found it is about 2 μM in vitreous fluid [18]. Notably, no nitrite peak was observed in any of our samples confirming that the level is low.

4. Conclusions

In this work, a useful quantification method is described for the analysis of nitrate levels in the vitreous of patients undergoing vitrectomy for PDR compared to those undergoing vitrectomy for MH and ERM. Furthermore, the effect of the complex biological matrix is minimized with the standard addition method giving good agreement with other reports of nitrate levels. Nitrate is stable in the acetic acid diluted vitreous for periods up to a month. Importantly, the stability of the analyte allows the assay setup to be distant from source of vitreous samples. Only a small sample volume is required; and there are other unknown peaks detected from the vitreous, which might allow this method to be used for further analysis of other important markers. It is concluded that our CE determination method is a useful and fast (<5 min) way to detect NO_3^- from human vitreous.

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